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Rho-independent termination: dyad symmetry in DNA causes RNA polymerase to pause during transcription in vitro

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ABSTRACT

Termination of transcription by RNA polymerase at rho-independent sites appears to depend primarily upon two structural features, a region of GC-rich dyad symmetry in the DNA preceding the stop point and a stretch of uridines at the 3' end of the transcript. The possibility that the former might be responsible for slowing elongation prompted us to perform a kinetic analysis of transcription across the leader and terminator regions of the E. coli tryptophan (trp) operon. Regions where the elongation rate is dramatically slowed or stopped are identifiable because they generate discrete transcript bands on a gel. Species derived from pause sites, unlike those resulting from termination sites, are transient and detectable only within the first two minutes of transcription, since polymerase eventually resumes elongation. At two mutant trp attenuator sites (trp al35 and trp al419), where termination is incomplete or absent in vitro, a substantial pause is nevertheless observed. Likewise, a significant pause occurs at trp t, the termination site at the end of the operon. Our experiments also reveal a major pause site at about position 90 in the trp leader sequence, just past a region of dyad symmetry. The RNA hairpin corresponding to this site is U-rich, and pausing is strongly enhanced by incorporation of BrUTP. In contrast, this analog does not affect pausing at the attenuator or terminator sites with hairpins that are GC-rich. These results strongly support the hypothesis that pausing of the polymerase is an obligatory prelude to rho-independent termination. Moreover, the termination event evidently results from consecutive but discrete responses to separate structural features of these sites.

INTRODUCTION

Transcriptional termination events fulfill several important roles in the cell. At the ends of genes and operons, transcription termination prevents interference with expression from neighboring regions of the chromosome. Termination also occurs near the beginning of many gene clusters at attenuator sites, to control transcription by regulating the number of RNA polymerase molecules that transcribe distal structural genes. Termination by RNA polymerase may occur directly in response to a nucleotide sequence or structure, or require additional factors for efficient action. Studies on mechanisms of termination are somewhat complicated by

the fact that RNA polymerase may behave quite differently at a particular site *in vivo* and *in vitro* (1-4). Nevertheless, common features of terminator regions have been identified (5,6) including a GC-rich region of dyad symmetry preceding the termination site and a series of uridine residues at the 3' terminus of the RNA transcript.

A major advance in understanding came with the realization that these two features reflect the involvement of both RNA-RNA and RNA-DNA interactions, and that RNA polymerase requires both a hairpin structure in the RNA as well as the subsequent sequence of uridines in order to terminate at rho-independent sites (5-9). We suggest that an RNA polymerase molecule newly arrived at a termination region is in an "elongation" configuration, and may pause on the template but is still capable of continued polymerization. However, in response to some feature at the termination site the polymerase probably undergoes a conformational change and becomes unable to add another nucleotide to the nascent RNA, thus forming a termination complex (9). Release of the transcript is eventually followed by dissociation of the polymerase-DNA complex.

Previous work has indicated that rho is involved as a release factor for termination *in vitro*, in rho-independent as well as rho-dependent systems (10-13). The primary function of rho may be to interact with termination complexes that have ceased elongation, and to increase their rate of dissociation. At the rho-dependent terminator lambda t_{R1}, in the absence of rho, polymerase pauses at the site where it would normally terminate, before continuing readthrough polymerization (14). RNA polymerase has also been found to hesitate at other termination sites in the absence of rho (15-18). The ability of the polymerase to pause at a specific site may be due to a GC-rich region upstream from a termination site which can impede the progress of the polymerase (19), or the hairpin structure in the RNA might interact with the RNA polymerase thus causing it to slow down (5).

In an attempt to clarify the role of pausing in termination, we have performed a kinetic analysis of transcription from the leader and terminator regions of the E. coli *trp* operon, and examined the behavior of normal and altered termination sites. We have found that in some cases significant pausing of the polymerase on the transcript can occur without resulting in termination. In addition, the use of base analogs for transcription *in vitro* has allowed us to define the character of these sites more precisely. Our results suggest a definite role for each of the two

predominant structural features of termination sites, and we propose that the molecular events leading to rho-independent termination are discrete and well-ordered.

MATERIALS AND METHODS

Most enzymes and plasmid strains have been described (9,20), the wildtype 560 bp HpaII fragment and the trp al419 510 bp HinfI fragments are shown schematically in Figure 1a and 1b. The trp al35 HpaII 560 bp fragment is identical to that of wildtype template except for a U to G base change at position 135 (designated trpL135) and a G to A base change at position 75 (designated trpL75). The plasmid from which this fragment was derived was the generous gift of C. Yanofsky. Figure 1c shows an 800 bp HaeIII fragment isolated from pWU11 carrying the trp promoter adjacent to trp t (the termination site immediately following trpA at the end of the operon; see 3,4).

Reactions were preincubated at 37° for 10 minutes in a total volume of 100 µl that was 200 µM in two unlabeled nucleoside triphosphates, 10 to 20 µM in the third unlabeled triphosphate, 20 mM Tris-acetate (pH 7.9), 0.1 mM Na_2EDTA, 0.1 mM dithiothreitol, 150 mM KCl and contained 40 µCi of [α^{32}P]-labeled GTP to give a final concentration of 10-20 µM, 1.0 pmoles template, and 2 µg (1 pmole) of RNA polymerase. The concentration of one unlabeled triphosphate was dropped below that of the others in order to slow the reaction enough so that time points at 15 second intervals could be taken. As a control we performed several experiments with all three unlabelled triphosphates at 200 µM, and the labelled nucleotide at 10-20 µM; though transcription proceeds faster, the qualitative results do not differ from those obtained with the slightly limiting conditions.

To synchronize initiation and limit transcription to one round of synthesis after preincubation, $MgAc_2$ and rifampicin were added simultaneously, to 4 mM and 10 µg/ml respectively. After initiation, 10 µl aliquots were removed at 15 to 30 second intervals and put directly onto dry ice; 100 µl of 0.3 M sodium acetate, 1 mM EDTA, 0.5 mg/ml tRNA were then added to each tube. Thawed samples were then phenol extracted, ethanol precipitated, and analyzed by gel electrophoresis (in tris-borate-EDTA) on 5% or 10% acrylamide gels, 7M in urea as described previously (11). The base analog BrUTP was a gift from D. Ward and used in place of UTP at 200 µM final concentration; the elongation rate was not detectably affected by the substitution of this analog (data not shown).

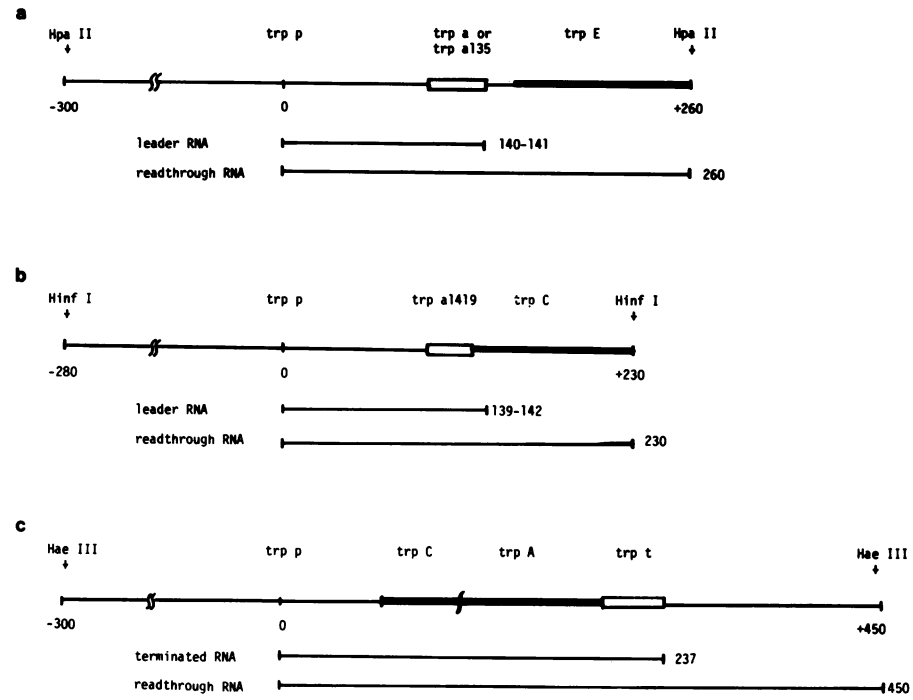


Figure 1. Comparison of the trp DNA templates. The DNA fragments shown were isolated and used as templates for transcription in vitro (see Materials and Methods). The promoter, trp p, is responsible for almost all transcription initiation under the conditions used; numbers indicate distance from the startpoint of transcription. The products of transcription from each fragment are the terminated RNAs (terminating at either the trp attenuator or trp terminator) and readthrough RNAs. In the wild-type DNA, trp a precedes the trpE structural gene by about 20 nucleotides. The trp a135 DNA is idential to wildtype except for a G to A change at position 75 (not shown) and a U to G change in the attenuator region at position 135. In the template carrying the trp a1419 deletion, the trp attenuator is fused to the trpC sequence about 150 nucleotides from the distal end of that gene. The template carrying trp t consists of a fusion of two fragments, one carrying the trp promoter joined to trpC (by an internal deletion) and the other carrying the end of trpA and extending beyond trp t.

RESULTS

Previous analysis of an altered trp attenuator site (trp a1419) has shown that termination can be abolished by a mutation which does not affect the strong region of dyad symmetry preceding the point of termination (21,9). However, preliminary kinetic experiments utilizing this

template indicated that RNA polymerase may pause for significant lengths of time at the mutant attenuator site without actually terminating. We have now analyzed the kinetic behavior of RNA polymerase on this and three other templates, which are schematically shown in Figure 1. The E. coli trp attenuator site normally functions in vitro as an efficient terminator without added factors, and resembles other terminators in that the transcript has a termination stem containing seven base-pairs followed by eight uridines (stem 3:4 in Fig. 2a); mutations in this region can modify termination efficiency. We examined transcription of two mutant templates as well as wildtype. The mutation trp al35 is a U to G change at position 135, which increases the length of the termination stem and decreases the number of consecutive uridines to 6 (see Fig. 2); readthrough transcription in vitro is increased to 50% compared to the 5% seen with the paren-

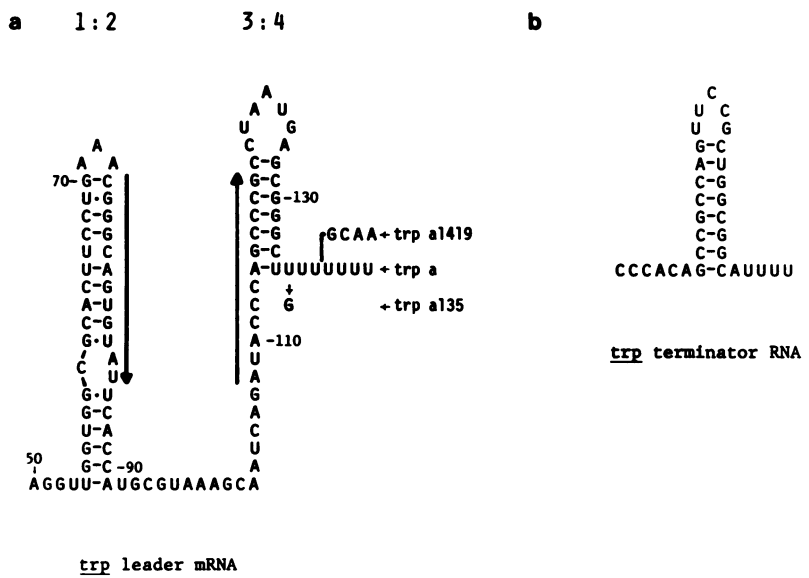


Figure 2. RNA sequence of the trp attenuator and terminator regions. The DNA sequences of the leader region (21), across the trp al419 junction (9), the point mutation trp al35 (20), and the trp terminator sequence (3, 22) have been reported. Here we show the potential hairpins which can form in the transcripts from the templates in Figure 1. Transcription termination occurs after either the 7th or 8th uridine in the trp attenuator region and after the 4th uridine of the trp terminator. The arrows represent another stem and loop (2:3) which is thought to form only when transcription occurs in vivo. The trp al35 mutation is the U to G base change shown at position 135; the trp al419 deletion causes replacement of the last four uridines in the transcript by trpC sequence, as shown.

tal L75 template (20). The deletion that generated trp al419 removed the last four TA base pairs of the wildtype attenuator in addition to the distal AT rich sequence, and causes 100% readthrough in vitro (21, 9). The fourth template we utilized (Fig. 1c) carries the terminator site at the end of the trp operon (3, 22). The resulting transcript has a hairpin with an eight base pair stem (seven of which are GC's) followed by one A and four U's (Fig. 2b). This terminator has been found to be about 25% efficient and independent of rho factor in vitro (4).

For all the kinetic experiments described below, transcription was synchronized and limited to one initiation event (see Materials and Methods). Analysis of samples taken at various times after initiation revealed the progress of polymerase along the template.

I. Pause Reaction on the trp al419 Template. Although termination does not normally occur in vitro at this mutant attenuator (21, 9), RNA polymerase does pause briefly at the trp al419 stem (3:4) before continuing to produce full readthrough transcription (Fig. 3). If the polymerase, in fact, did not respond at all to the altered attenuator (as initial results suggested), then each time point would exhibit a random collection of

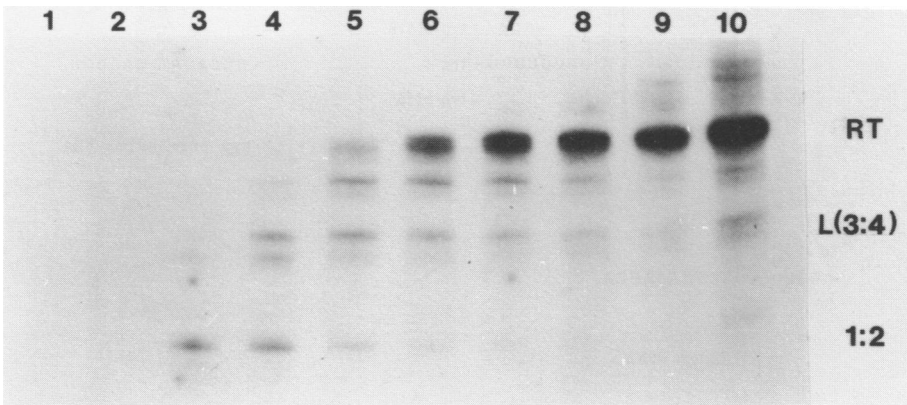


Figure 3. Pause Reaction on trp al419 Template. Lane 1 is a control lane, taken before $MgAc_2$ and rifampicin were added. Lanes 2-8 represent aliquots taken at 15 second intervals after the addition of $MgAc_2$ and rifampicin, whereas lanes 9 and 10 are 30 second intervals. It can be seen that the polymerase remains at the termination region (L) before continuing the readthrough transcription (RT). The small molecular weight band is a pause site at stem 1:2 in the leader region. The high molecular weight transcripts seen between the attenuator and the end of the fragment could be due to the extensive dyad symmetry in the DNA.

bands. The discrete series of bands actually observed suggests that the polymerase molecule is retarded at precise locations on the template for varying times, with rapid elongation of transcription occuring between these sites. The bands which appear between the leader and readthrough bands are most likely due to the pausing of polymerase at regions in the sequence of trpC (see Fig. 1). The DNA of this portion of trpC is GC-rich and does contain extensive regions of dyad symmetry (23). The significance of these bands will be considered in the discussion, and the band representing the small molecular weight transcript (1:2) will be discussed in section III below.

II. Pause Reaction on the trp Terminator. Figure 4 shows the results of transcription from a template carrying the termination region of the trp operon (Fig. 1c and 2b). Since this template is longer than the trp attenuator templates, the time points were chosen so that the appearance and disappearance of the pause sites could be seen more easily on the gel. The lower band in Figure 4 (t) represents pausing of the polymerase at the trp terminator site. Although this site is only 25% efficient in termination (4), it is clear that polymerase remains at the terminator for more than 50 seconds before continuing the elongation process of readthrough transcription.

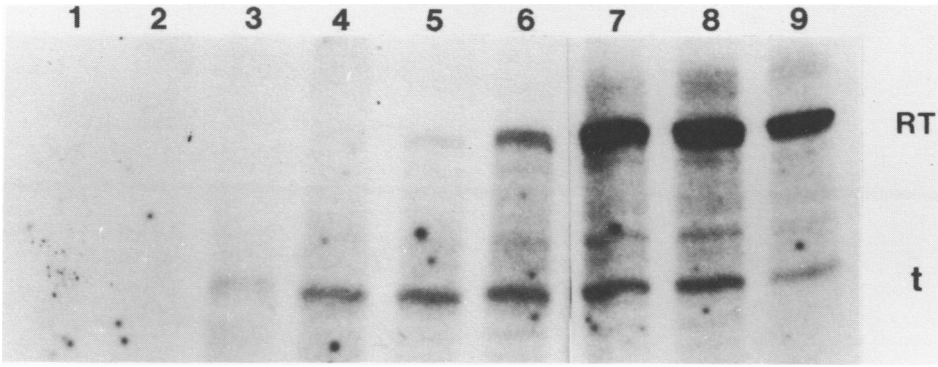
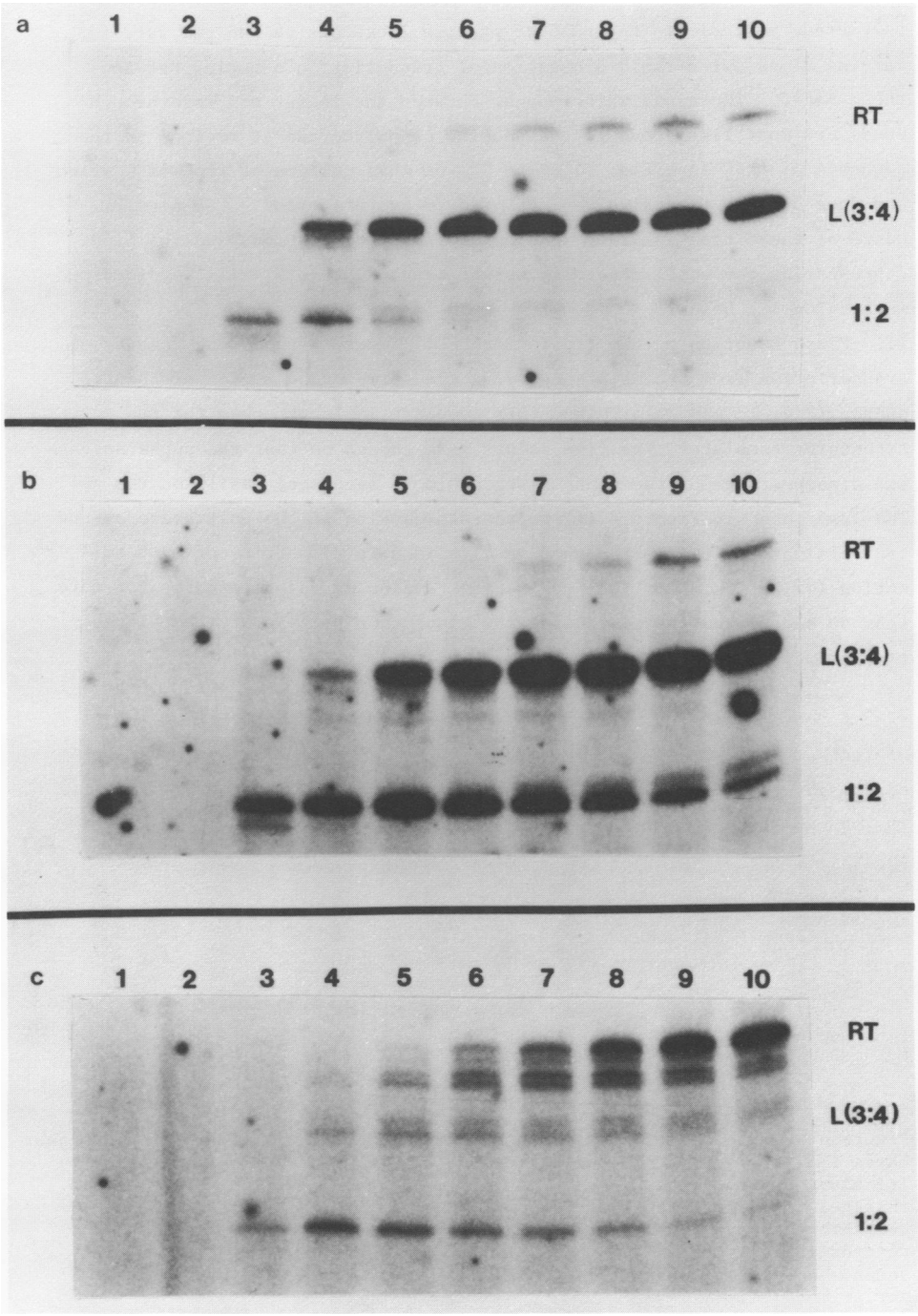


Figure 4. Pause Reaction on the trp Terminator. Lane 1 is the control lane. Lanes 2-6 represent 20 second intervals starting 30 seconds after initiation, whereas lanes 7-9 are at 30 second intervals. The two bands in this gel represent transcription ending at the trp terminator (t) and readthrough transcription (RT). It can be seen that the trp terminator retards the polymerase at the terminator region for approximately 50 seconds before allowing it to continue on to give 80% readthrough transcription.


a
1 2 3 4 5 6 7 8 9 10
RT
L(3:4)
1:2
b
1 2 3 4 5 6 7 8 9 10
RT
L(3:4)
1:2
c
1 2 3 4 5 6 7 8 9 10
RT
L(3:4)
1:2

III. The Effect of BrUTP on Pausing in the Leader Region. The results presented above illustrate that significant pausing can occur at inefficient termination sites, and suggest that other regions can also function as pause sites. Figure 5a shows pause reactions on the wildtype trp attenuator. As expected from in vitro reactions, transcription is terminated at the attenuator site except for the small amount of readthrough which occurs. However, a striking pause can be seen prior to the attenuator site; the transcript resulting from this pause gradually disappears, indicating that the polymerase eventually continues elongation. This same pause site is seen in Figure 3 on the trp al419 template. This RNA species was fingerprinted (data not shown) and found to terminate about position 90, on the distal side of stem and loop 1:2 in the trp attenuator region (Fig. 2). Winkler and Yanofsky have also observed this species and characterized its pausing behavior (24).

When BrUTP was incorporated into the transcript in the place of UTP, the amount of pausing was greatly enhanced at stem 1:2 (Fig. 5b and 5c). This stem differs from 3:4 in that it contains several AU and GU base pairs which would be strengthened upon substitution of BrUTP for UTP (see Fig. 2). As expected, no significant change occurs in the pause response at the GC-rich (3:4) stems of either the wildtype or trp al419 attenuators.

IV. Pause Reaction on trp al35 Template. The trp al35 mutation (Fig. 2a) results in an attenuator with a longer potential stem as well as a shorter stretch of consecutive uridines, and elevates readthrough transcription substantially (20). Our kinetic analysis (Fig. 6) demonstrates that in spite of the reduced termination, a pause is still present at the 3:4 site (lanes 4-6), and it is similar in duration to that seen at trp al419 (Fig. 3).

Figure 5. Effect of BrUTP on Pausing in the Leader Region. Lanes 1-10 represent the same time points as in Figure 3. Figure 5a demonstrates the pausing of the RNA polymerase on the wildtype trp attenuator region. The three bands correspond to the transcripts produced when the polymerase is stopped at stem 1:2, the attenuator stem (3:4) and the end of the fragment. The same transcripts are seen with the incorporation of BrUTP into the RNA. Figures 5b and 5c show that the band corresponding to stem 1:2 is present for a much longer time period in the presence of BrUTP, and though the intensity of the bands differ, the onset and duration of the pause is similar in both cases. At the same time, no change can be seen in the amount of pausing at either the wildtype attenuator (5b) or trp al419 (5c), indicating that the polymerase remains there for about the same length of time as in the previous reactions.

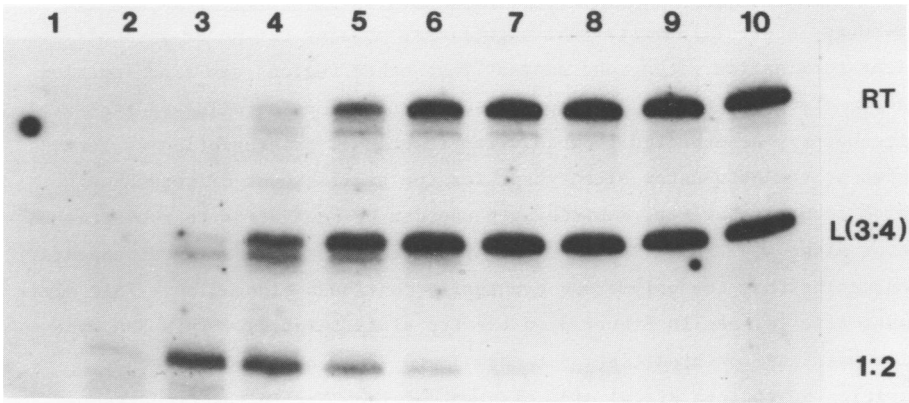


Figure 6. Pause Reaction on trp a135 Template. Lanes 1-10 represent the same time points as in Figure 3. It can be seen that the pause at stem 1:2 is unchanged by the point mutation at position 75. The effect at the attenuator site is, however, changed by the mutation at position 135. The mutant attenuator is much less efficient at termination, but can nevertheless significantly retard the polymerase at the attenuator region before allowing it to continue on to readthrough transcription.

DISCUSSION

The behavior of RNA polymerase at termination sites is subject to many variables, including the nucleotide sequence, mutations in RNA polymerase, incorporation of nucleotide analogs, and the participation of additional factors. We have been interested in dissecting the mechanism of termination, and recently provided evidence that each of the two main features common to rho-independent sites is important in termination, though they contribute in different ways (9). The few completely rho-dependent sites that have been analyzed are partially deficient in these properties. At the lambda t_{R1} terminator, although no termination is observable in the absence of rho factor, in vitro kinetic experiments demonstrated that elongation of RNA synthesis pauses at this site (14). Moreover, the introduction of point mutations that would disrupt the t_{R1} hairpin effectively eliminated the observable pausing (14).

We have performed similar time-course experiments on several templates derived from the leader and terminator regions of the E. coli trp operon, and our results support and extend the previous observations: that RNA synthesis in vitro may be discontinuous and can slow down or stop completely in response to particular sequences. Our findings demonstrate

that RNA polymerase pauses at several specific sites on different DNA templates. The common feature retained by these sites is a significiant stretch of dyad symmetry, permitting hairpin formation in the transcript. Although dyad symmetry is a common feature of termination sites, its function has not been clear. Our experiments support the hypothesis of Adhya and Gottesman (5), that it is the hairpin which causes RNA polymerase to pause on the template.

Our present observations show that RNA polymerase will pause at the trp a1419 attenuator (Fig. 2), although termination does not occur there at all in vitro. Since the trp a1419 template lacks the normal distal sequence, this result implies that some prior feature, which we believe to be the hairpin, is capable of causing polymerase to hesitate at the termination site. We presume that the absence of termination in this case is due to an inability to release the transcript from the temple (8,9), so that the polymerase therefore eventually continues transcription.

The trp terminator (trp t) resembles the trp a1419 attenuator site in that both have only four distaluridines (Fig. 2) and are inefficient in vitro, yet both contain strong hairpins. The trp t stem should therefore elicit a strong pause by RNA polymerase, which in fact, it does (Fig. 4). Furthermore, the longer hairpin at trp t (compared to that at trp a1419) correlates with a longer pause.

We have found as well that hairpins other than those normally involved in termination can also elicit a pause. Our observations of a long pause at the early (1:2) hairpin in the trp leader region (Figure 5a) are in agreement with those of Winkler and Yanofsky (24); the absence of a subsequent stretch of uridines in the RNA presumably prevents termination in a normal transcription system. The function of this early structure in the attenuation response in vivo may be to allow the distal (3:4) termination hairpin to form without interference in the absence of translation (25, 26).

The major evidence that the strength of base-pairing in the stem of these hairpins governs termination is provided from experiments utilizing base analogs, where those that stabilize pairing (such as BrUTP or ICTP) enhance termination, and those that destabilize pairing (such as ITP) enhance readthrough transcription (27-29, 9). Our results offer particular support for this idea with the incorporation of BrUTP into the trp a and trp a1419 transcripts: there is a striking effect on pausing at the early (1:2) leader site (which contains at least 7 potential pairs in-

volving uridine) but no detectable effect on the GC-rich termination sites (Fig. 5b and 5c). Thus the strength of the hairpin actually appears to affect the length of time that polymerase remains paused at the site.

Pause reactions on the trp a135 template qualitatively illustrate the effects of changing both the number of uridines and the stem length. The point mutation at position 135 decreases the number of uridines to six while increasing the length of the potential 3:4 hairpin. A comparison of these reactions to those with the wildtype template reveals that although a long pause is also elicited by the trp a135 hairpin, greater readthrough eventually occurs (Fig. 5a and 6). We ascribe the substantial pause to a strong hairpin, and the greater readthrough to a reduction in the number of uridines.

Our recent model predicts that termination at rho-independent sites results from the combination of two separate events. Elongation ceases first, and a subsequent response in the transcription complex allows the RNA to be released. The fact that pausing can occur at hairpins which are not followed by the appropriate stretch of uridines implies that whether or not a polymerase hesitates at the termination site is determined by the stem itself, regardless of the stability of the RNA-DNA interactions immediately following the hairpin. The contribution of the uridines is inferred from the observations that BrUTP incorporation enhances readthrough at the trp attenuator (9), but does not decrease pausing at this site (Fig. 5b and 5c).

The distinction between pausing and termination can be seen in the studies on wildtype and mutant attenuators. Though pausing cannot be studied at the wildtype trp attenuator site because termination is complete (30), when some of the uridines are removed from the transcript by mutation, this site loses its termination efficiency but retains pausing ability (Fig. 3). These observations are consistent with a model that the complete termination event requires both hairpin formation in the transcript to elicit a pause, and a series of uridines to facilitate release.

The demonstration that RNA polymerase can pause at regions on a DNA template that normally do not function as termination sites is complemented by the observations of Mills et al. (31) that transcription by Qβ replicase on an RNA template also pauses at points where the product strand can form a terminal hairpin. It is thus apparent that hairpin structure may be involved in a general mechanism for regulating the elongation rate across regions of biological importance, and that this may

have important implications for related aspects of transcriptional regulation. For example, a possible additional function of the early (1:2) hairpin in the trp leader region is to retard the polymerase in order to permit the translating ribosomes to catch up and remain coupled to the transcription apparatus. The potential significance of this tight coupling for the attenuation response is discussed by Yanofsky (26). The presence of dyad symmetry within structural genes may provide for pausing and the formation of rho-dependent termination complexes by RNA polymerase in the absence of translation, and account for premature transcription termination in polarity (5). Calva and Burgess (32) have discovered a rho-dependent termination site within the lambda cro gene which is consistent with this mode of operation; so is the pausing at sites of symmetry late in trpC that we observe in the trp al419 template (Fig. 3). Another potential use for hairpin structures is to help mediate interactions between RNA polymerase and other proteins such as antitermination factors. For example, the symmetric structure of the nut sites in lambda suggests that polymerase may pause there, possibly to "load" N-protein (see 5); such a mechanism would be applicable to other regulatory macromolecules as well.

In summary, current studies on the mechanism of transcription termination are consistent with the idea that dyad symmetry sufficient for stable hairpin formation in the transcript, followed by a number of consecutive uridine residues, will cause RNA polymerase to cease elongation in the absence of additional factors in vitro. The contribution of these two features is becoming more clear: an RNA hairpin induces the polymerase to slow or stop chain elongation and thus increase the opportunity for termination, then a change in polymerase configuration together with the run of uridines stimulates dissociation of the transcript from the template, preventing further elongation (though immediate dissociation of the ternary complex is probably not obligatory). The precise nature of the interaction between the RNA transcript and the polymerase molecule is still completely obscure, and the behavior of the polymerase at sites lacking the uridine residues must be responsive to other factors in the cell, such as translation of the mRNA, or the presence of rho protein or antitermination molecules. The potential flexibility that these variations introduce, however, increases the subtlety with which RNA synthesis can be regulated after the initiation of transcription.

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